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Extraction of a Vanadium-Binding Substance (Vanadobin) from the Blood Cells of Several Ascidian Species

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Abstract. A combination of techniques, including chromatography on Sephadex G-15 and SE-cellulose columns and neutron activation analysis for vanadium determination, was used to extract (at low pH) a vanadium-binding substance (vanadobin) from the blood cells of the ascidian species: *Ascidia ahodori* OKA, *A. gemmata* SLUITER, *A. zara* OKA, *Corella japonica* HERDMAN, and *Ciona intestinalis* (LINNE). In general, ascidians can be classified into two different categories based on vanadium content: species of the family Ascidiidae contain high levels of vanadium, whereas those in the Cionidae and Corellidae do not always have such high amounts. Because *Ciona intestinalis* and *Corella japonica* do have vanadobin in their blood cells, vanadobin may well be a universal complex in ascidians, having the role of accumulating vanadium in blood cells and maintaining its concentration. The blood cells of *A. gemmata* contained the highest amount of vanadium. Vanadobin extracted from these cells exhibits absorption spectra, not only in the ultraviolet region, but also in the visible region; such spectra correspond to those observed in vanadium complexes in oxidation states of +3 and +4.

Introduction

The unusual ability of ascidian blood cells to accumulate vanadium in excess of one million times its level in seawater has attracted the interest of investigators from various fields of study. In particular, the chemical form of the vanadium complex present in ascidian blood cells has long been a subject of discussion. Sometime after Henze's first discovery of vanadium in ascidian blood cells (Henze, 1911), the element was believed to occur as part of a sulfated nitrogenous compound known as haemovanadin (Califano and Boeri, 1950; Webb, 1956; Bielig *et al.*, 1966). Kustin's group claimed that haemovanadin is an artificial product generated by air oxidation (Kustin *et al.*, 1976; Macara *et al.*, 1979a, b).

The vanadium ion dissolved in seawater seems to exist as the vanadate(V) anion in the +5 oxidation state (McLeod *et al.*, 1975), but this is still experimentally unresolved (Biggs and Swinehart, 1976). The vanadium ion contained in ascidian blood cells is, however, reduced to +4 or +3 oxidation states, *i.e.*, vanadyl cations (*cf.* Michibata and Sakurai, 1990). It has therefore been assumed that agents causing the reduction of vanadate ion to vanadyl ion must occur within ascidian blood cells and there bind with the vanadium ion. Kustin's group isolated a tunichrome from ascidian blood cells: it was proposed as being involved in the accumulation of vanadium and in its reduction from seawater (Macara *et al.*, 1979a, b; Bruening *et al.*, 1985). However, there is still no evidence that this tunichrome fulfills those functions in vanadium-containing blood cells (vanadocytes). Furthermore, the fluorescence due to the tunichrome is certainly not detected in the signet ring cells that have been identified as the vanadocytes (Michibata *et al.*, 1988, 1990a).

While extracting tunichrome, Gilbert *et al.* (1977) and Macara *et al.* (1979a) found a vanadium-containing band upon Sephadex column chromatography, but they continued to focus mainly on characterizing the tuni-

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chrome. More recently, in contrast, while characterizing a vanadium-binding substance extracted from the blood cells of *Ascidia sydneiensis samea* OKA under acidic conditions, we showed that this substance, named vanadobin, could maintain the vanadium ion in the vanadyl form (VO(IV)), had an apparent affinity for vanadium ion, and contained a reducing sugar (Michibata *et al.*, 1986a).

The present investigation was designed to determine whether vanadobin could be extracted from the blood cells of other ascidians that contain significant amounts of vanadium and, thus, to ascertain whether vanadobin is a universal characteristic of blood cells in vanadium-containing ascidians. In fact, vanadobin was extracted from the blood cells of all ascidian species examined. Furthermore, the vanadobin extracted from *A. gemmata* shows an absorbance in the visible range resembling that of a vanadium compound.

Materials and Methods

Ascidia ahodori OKA, *A. zara* OKA, and *Ciona intestinalis* (LINNE) were collected from the Ushimado Marine Biological Station of Okayama University in Ushimado, Okayama Prefecture, Japan. *A. gemmata* SLUITER was obtained from the Asamushi Marine Biological Station of Tohoku University in Asamushi, Aomori Prefecture. *Corella japonica* HERDMAN was gathered in Yamada Bay near the Ootsuchi Marine Research Center, Ocean Research Institute of the University of Tokyo in Ootsuchi, Iwate Prefecture.

Blood from each species was collected by cardiac puncture under an anaerobic atmosphere of nitrogen gas to preclude air-oxidation; subsequent techniques were also carried out under the same conditions. The blood cells were separated from the blood plasma by centrifugation at $3000 \times g$ for 20 min at 4°C, and were pooled at −80°C before use. A 10 m*M* glycine-HCl buffer solution at pH 2.3 was added to the cell pellet, and the suspension was then ground in a glass-Teflon homogenizer at 4°C. For *A. gemmata* and *Corella japonica*, glycine-HCl buffer was substituted with a 50 m*M* HCl solution throughout the experimental process.

The homogenate was loaded onto a column of Sephadex G-15 (Pharmacia Fine Chemicals). The column size and the chromatographic parameters used with each species is described in the appropriate figure legend. The column was equilibrated with the glycine-HCl buffer solution, and the elutant was collected in 3 ml or 5 ml fractions with monitoring for UV absorbance.

The amounts of vanadium in each blood cell pellet and in the fractions were measured by neutron activation analysis or atomic absorption spectrometry. For neutron activation analysis, the elutant of each fraction was repacked in a polyethylene capsule and irradiated with thermal neutrons having a flux of 5×10^{11} n/cm$^2 \times$ s^{-1} for 2 min in a TRIGA MARK II nuclear reactor at the Institute for Atomic Energy, Rikkyo University, Yokosuka, Japan. The radioactivity of ^{52}V produced in the irradiated sample was measured with a 50-cm^3 Ge(Li) γ-ray spectrometer (Canberra Inc.) 2 min after the irradiation. Using a photon energy for ^{52}V of 1432KeV, the amount of vanadium in the sample was determined by comparison with that of a standard, as described previously (Michibata *et al.*, 1986b). Flameless atomic absorption spectrometry was applied to the samples that contained relatively higher amounts of vanadium. For these measurements we used a Hitachi GA-2 with a graphite furnace, and an absorption line of 3183.9 A was used for vanadium determination.

The absorbance of fractions observed to contain vanadium was measured with a spectrophotometer (Hitachi U-3210); the fractions were then pooled and loaded onto a column of SE-52 (Serva Feinbiochimica) for ion-exchange chromatography (Whatman W. & R.) and, thus, for further purification. Non-absorbed substances could be washed off the column with a sufficient volume of 10 m*M* glycine-HCl buffer, and the purer vanadobin could then be eluted with a linear gradient of KCl, from 0 to 0.5 *M* in the buffer solution. The vanadium content in each fraction obtained was also measured by the methods described above, and absorbance was monitored with a spectrophotometer.

We examined whether a simulated absorption spectrum of vanadobin from *A. gemmata* could be reproduced using spectra of inorganic vanadium complexes. Vanadium(III) sulfate ($V_2(SO_4)_3$) was dissolved in 30 m*M* H_2SO_4, in a concentration of 40 m*M* at pH 1, under an anaerobic atmosphere of argon gas, and vanadium(IV) oxide sulfate ($VOSO_4$) was dissolved in distilled water (10 m*M*, at pH 2.0 to 3.5 adjusted with 1 *M* HCl). Each absorbance was measured with a spectrophotometer, and each molar extinction coefficient was calculated. Based on these data, a spectrum closely resembling that of vanadobin was calculated.

Results

Ascidia gemmata *SLUITER*

This species contained a higher level of vanadium in its blood cells than any other species examined (8.75 μg/mg wet weight) (Table I). Figure 1 shows the elution profile that resulted when a homogenate of the blood cell pellet (1.64 g wet weight) of *A. gemmata* was loaded onto Sephadex G-15 and eluted. A large peak in fractions 8 through 22 was observed. As shown in Figure 2, the spec-

Table I

Extraction process of vanadobin from the blood cells of several ascidian species through chromatography

	Pellet of blood cells		Sephadex G-15		SE-cellulose	
	Wet weight (mg)	V content (μg)	V content (μg)	Abs. max. (nm)	V content (μg)	Abs. max. (nm)
Ascidia gemmata	1640.0	14,350.0 (100.0%)	13,043.5 (91.0%)	245, 410, 756	8347.8 (64.0%)	238, 756
Ascidia ahodori	49.7	101.4 (100.0%)	62.5 (61.6%)	254	25.9 (25.5%)	232
Ascidia zara	40.1	140.5 (100.0%)	30.6 (21.3%)	236		
Corella japonica	13,900.0	269.0 (100.0%)	236.0 (87.7%)	262	222.8 (82.8%)	260
Ciona intestinalis	86.3	22.5 (100.0%)	6.9 (30.7%)	246		

V content: vanadium content, Abs. max.: absorption maximum. The recovered vanadium through the chromatography is expressed as percent of the initial amount in the pellet of blood cells in parentheses.

trum of the peak fraction (fraction 13) had a clear ultraviolet peak at 245 nm, and absorption peaks at 410 nm and 756 nm in the visible range with a shoulder at 620 nm. The elution profile of vanadium coincided with the peak of absorbance. The total vanadium content detected in fractions 8 through 20 was 13043.5 μg.

The fractions from Sephadex G-15 column chromatography that contained vanadium were loaded onto a column of SE-52, providing the profile shown in Figure 3. By eluting with a gradient of KCl, several small peaks containing no vanadium were washed off the column, and then a sharp peak containing vanadium was obtained in fractions 199 through 216. The amount of vanadium recovered was 8347.8 μg. The peak fraction (fraction 205) exhibited absorption maxima at 236 nm and 756 nm, as shown in Figure 4. *A. gemmata* was the only species in which vanadobin exhibited absorption peaks in the visible range.

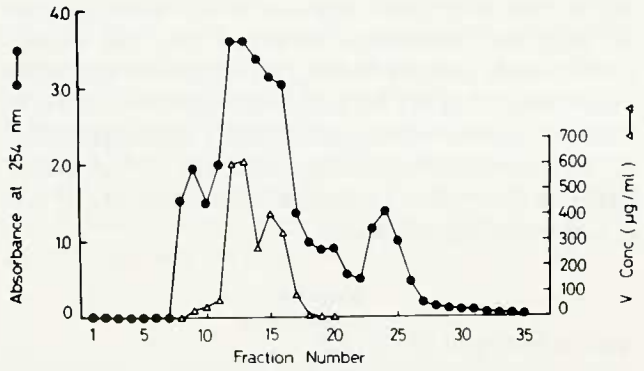

Figure 1. Elution profile of the blood cell homogenate of *Ascidia gemmata* on Sephadex G-15 column chromatography. Pelleted blood cells (1.64 g) were homogenized in about 50 m*M* HCl buffer solution, which was adjusted at pH 2.3. The homogenate (14 ml) was loaded onto a column (1.5 cm $\phi \times 48$ cm) and eluted with the same solution in 5-ml fractions. The total bed volume (Vt) and the void volume (Vo) of the column were 85 ml and 39 ml, respectively. The elution volume (Ve) of vanadobin was 65 ml.

Absorption spectra of vanadium(III) sulfate and vanadium(IV) oxide sulfate

Absorption spectra of vanadium(III) sulfate and vanadium(IV) oxide sulfate are shown in Figures 5a and b, respectively. The former had absorption peaks at 400 nm and 610 nm, and the latter a peak at 760 nm with a shoulder at 625 nm. When vanadium(III) sulfate ($V_2(SO_4)_3$)

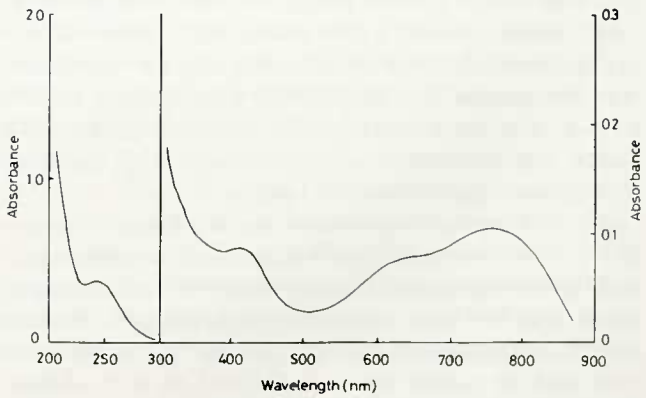

Figure 2. Absorption spectrum of vanadobin eluted from Sephadex G-15 column chromatography. The spectrum was recorded for the peak fraction 12 shown in Figure 1. Absorbance at 245 nm, 410 nm, and 756 nm with a shoulder 620 nm was observed.

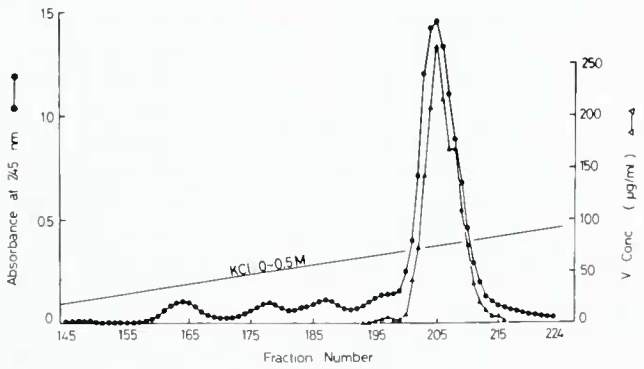

Figure 3. Elution profile of the vanadium-containing substance (vanadobin) of *Ascidia gemmata* on SE-cellulose column chromatography. Vanadium-containing fractions (45 ml) obtained after passing through Sephadex G-15 were loaded onto a column (3.7 cm $\phi \times$ 25 cm). After non-absorbed substances were washed off the column by eluting with 50 m*M* HCl solution at pH 2.3 in 5 ml fractions, the vanadobin was obtained by elution with a linear gradient of KCl at a concentration of 0.25 *M*.

and vanadium(IV) oxide sulfate ($VOSO_4$) were mixed together at concentrations of 4.8 m*M* and 5.9 m*M*, respectively, an absorption spectrum closely resembling that of vanadobin after elution through Sephadex G-15 (Fig. 2) was obtained, as shown in Figure 5c. This simulation clearly suggested that the vanadobin of *A. gemmata*, which was eluted from Sephadex G-15, contained vanadium in the +3 and +4 oxidation states in the ratio of 45:55, whereas for the purer vanadobin eluting from SE-cellulose, the absorption peak at 410 nm disappeared, and a peak at 756 nm with a shoulder at 620 nm was seen (Fig. 4). These findings indicate that, although vanadobin originally contains both vanadium forms in the +3 and +4 oxidation states, vanadium in the +3 oxidation state becomes oxidized to the +4 oxidation state during the purification.

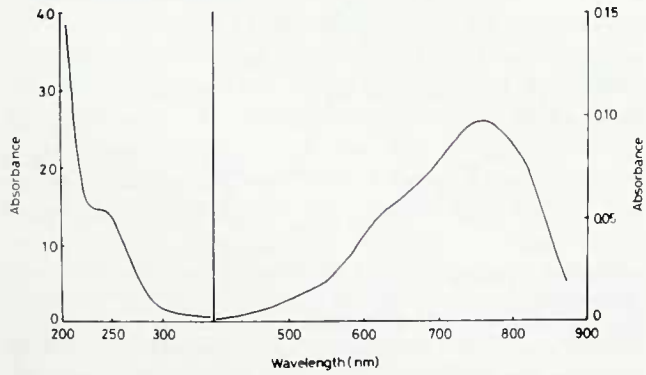

Figure 4. Absorption spectrum of vanadobin eluted from SE-cellulose column chromatography. The spectrum was recorded for the peak fraction 205 shown in Figure 3. Absorbance at 238 nm and 756 nm was observed.

Ascidia ahodori *OKA*

When a homogenate containing 49.7 mg wet weight of the cell pellet was loaded on the Sephadex G-15 col-

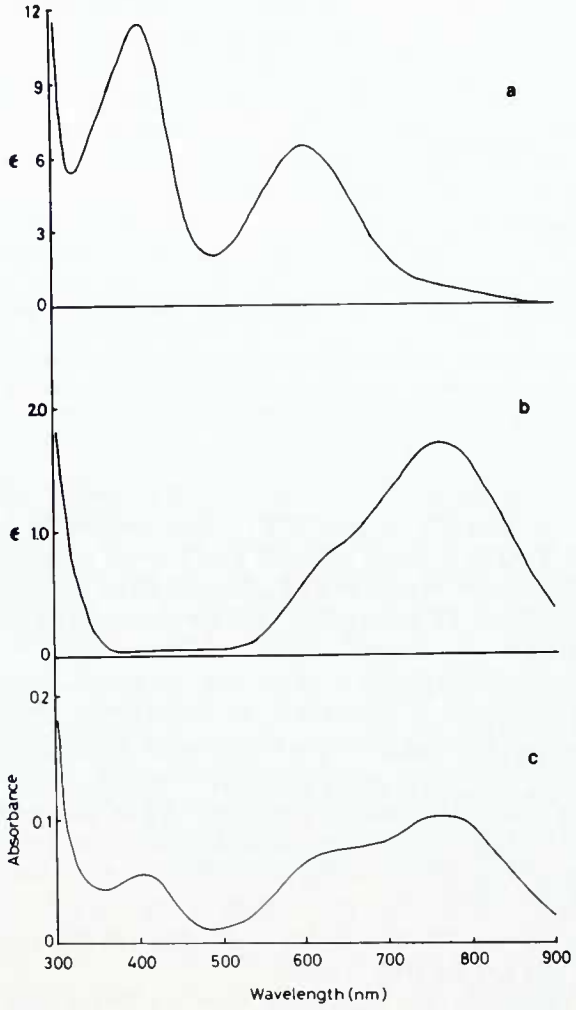

Figure 5. Absorption spectra of vanadium complexes. a. Vanadium(III) sulfate ($V_2(SO_4)_3$) was dissolved in 30 m*M* H_2SO_4 in a concentration of 40 m*M* at pH 1 under an anaerobic atmosphere of argon gas. Absorbance is expressed as (molar extinction coefficient). b. Vanadium(IV) oxide sulfate ($VOSO_4$) was dissolved in distilled water in a concentration of 10 m*M* at pH 2.0 to 3.5. Absorbance was expressed as (molar extinction coefficient). c. Simulated spectrum, closely resembling that of the vanadobin shown in Figure 2, was obtained when vanadium(III) sulfate ($V_2(SO_4)_3$) and vanadium(IV) oxide sulfate ($VOSO_4$) were mixed together at respective concentrations of 4.8 m*M* and 5.9 m*M*. This simulation suggested clearly that the chemical forms of vanadium in the +3 and +4 oxidation states were approximately in the ratio 5:6 in the vanadobin of *A. gemmata* eluted from Sephadex G-15 column (Fig. 2).

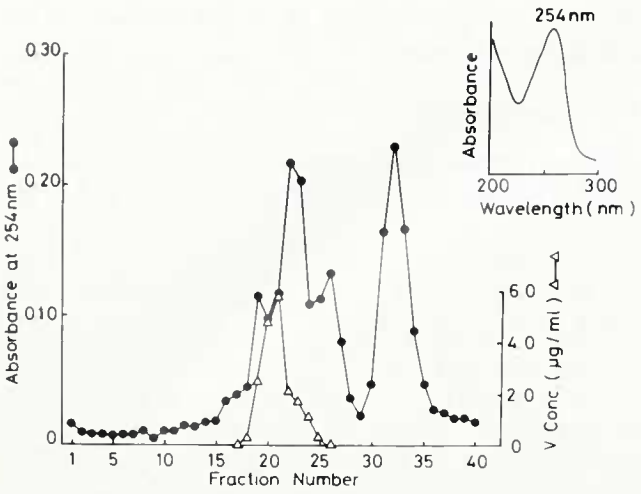

Figure 6. Elution profile of the blood cell homogenate of *Ascidia ahodori* on Sephadex G-15 column chromatography. Pelleted blood cells (49.7 mg) were homogenized in 10 m*M* glycine-HCl buffer solution at pH 2.3. The homogenate (1.2 ml) was loaded onto a column (1.5 cm $\phi \times 48$ cm) and eluted with the same buffer solution in 3 ml fractions. Vt and Vo of the column were 85 ml and 39 ml, respectively. Ve of vanadobin was 63 ml. The inset shows the UV spectrum of the peak fraction (fraction 21). Absorbance at 254 nm was observed.

umn, the elution profile obtained was somewhat more complicated (Fig. 6). A scan of UV wavelengths for fraction 21 (Fig. 6, inset) indicates that 254 nm is the peak of absorption. A total of 62.5 μg of vanadium was eluted in fractions 17 through 26, and its peak was in fraction 21.

As shown in Figure 7, when these vanadium-containing fractions were loaded onto a column of SE-52, non-absorbed substances were first washed off the column by eluting with the glycine-HCl buffer solution, and a big peak with no vanadium was eluted with a linear gradient of KCl dissolved in buffer solution. The vanadobin was obtained thereafter in fractions 54 through 58 with a peak in fraction 56. The vanadium content recovered was 25.9 μg. The absorption peak, observed in fraction 56, was 232 nm (Fig. 7, inset).

Vanadobin was extractable from the blood cells of three other ascidian species, *A. zara* OKA, *Corella japonica* HERDMAN, and *Ciona intestinalis* (LINNE); the procedures and results were similar to those described above (data not shown). The process of vanadobin extraction by chromatography is summarized in Table I. The highest vanadium content of 8750 ng/mg wet weight (14350 ng/1640 mg wet weight) in the blood cells was observed in *A. gemmata*. The next highest value was 2040 ng/mg wet weight detected in the blood cell pellet from *A. ahodori*. The blood cells of *Ciona intestinalis* had a much smaller content of 261 ng/mg wet weight of vanadium. *Corella japonica*, a vanadium-poor species, contained the smallest amount in its blood cells 19 ng/mg wet weight). Recovery rates of vanadium ion in fractions eluted through Sephadex G-15 were in the range of about 21–90%. The rates through SE-cellulose were in the range of about 26–83% of each initial amount. Peak wavelengths of UV absorbance in the peak fraction of Sephadex G-15 were observed between 246 nm and 262 nm, and the values observed in the peak fraction of SE-cellulose were shifted to shorter wavelengths, specifically to about 232 nm.

Discussion

Previous observations notwithstanding (Michibata *et al.*, 1986a), our present experiments show that vanadobin extracted from ascidian blood cells does, in fact, exhibit clear absorption peaks in the visible range when its contained vanadium in vanadobin is at a significantly high concentration. In the case of *A. gemmata*, the concentrations of vanadium in the peak fractions eluted from Sephadex G-15 and SE-cellulose columns were estimated to be 12.3 m*M* and 5.3 m*M*, respectively. The successful detection of this visible absorption is due to the high level of vanadium in the blood cells of *A. gemmata* than any other ascidian, and to the extremely large volume of sample loaded onto the column (*cf.* Table I).

Because the absorption spectrum of vanadobin from *A. gemmata* resembled those of vanadium compounds, an attempt was made to obtain a simulated spectrum.

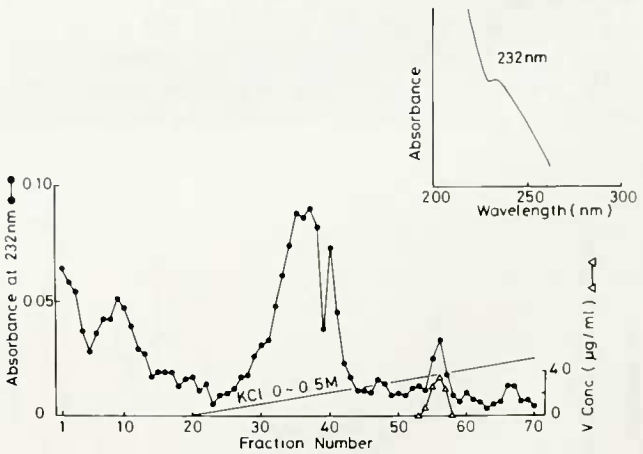

Figure 7. Elution profile of the vanadium-containing substance (vanadobin) of *Ascidia ahodori* on SE-cellulose column chromatography. Vanadium-containing fractions (15 ml) obtained after passing through Sephadex G-15 were loaded onto a column (1.2 cm $\phi \times 12$ cm). After non-absorbed substances were washed off the column by eluting with 10 m*M* glycine-HCl buffer solution at pH 2.3 in 3 ml fractions, vanadobin was obtained by elution with a linear gradient of KCl (0.1 *M*). The inset shows the UV spectrum of the peak fraction (fraction 56). Absorbance at 232 nm was observed.

Indeed, the spectrum of vanadobin eluted from Sephadex G-15 (Fig. 2) can be reproduced when vanadium compounds in the +3 and +4 oxidation states are mixed together in a ratio of 45:55 (Fig. 5c). We therefore conclude that vanadobin contains vanadium in the +3 and +4 oxidation states in this ratio. On the other hand, the peak at 410 nm disappears from vanadobin after elution through SE-cellulose (Fig. 4) because vanadium(III) is oxidized to vanadium(IV) during the experimental process.

The present report is the first to document an obvious absorption peak in the visible range for the native vanadium complex, vanadobin. Previously, an absorbance peak in the visible range at 430 nm had been reported for a blood cell lysate of *Ascidia obliqua* (Boeri and Ehrenberg, 1954); the 430-nm peak was attributed to the hydrolyzed ion $V(OH)_2V^{4+}$ (or VOV^{4+}). Brand *et al.* (1989) also found visible-range absorption of vanadium complexes from ascidian blood cells after addition of exogenous ligands, 2,2′-bypyridine, 1,10-phenanthroline, and acetylacetone.

Recent analysis of ascidian blood cells by NMR (nuclear magnetic resonance), EXAFS (extended X-ray absorption fine structure), and SQUID (superconducting quantum interference device) has suggested that the vanadium is present predominantly in the +3 oxidation state, and that the +4 state accounts for less than 10% (Carlson, 1975; Tullius *et al.*, 1980; Lee *et al.*, 1988). The present results confirm that data obtained from the visible spectrum are also available for further determination of the valency of vanadium in cases where a high concentration of the metal is present in ascidian blood cells.

The possibility has existed that vanadobin is an artificial complex produced during the experimental process. That is to say, although the signet ring cell, among several types of ascidian blood cells, is clearly the vanadium-containing blood cell (the vanadocyte) (Michibata *et al.*, 1987), the vanadium contained in the vanadocyte could well be mixed with a substance that was apt to bind with the metal contained in another cell type. Consequently, an artificial compound could be produced during homogenizing or chromatography. However, we had previously succeeded in excluding this possibility; vanadobin could be extracted from a homogenate of a pure subpopulation of signet ring cells (the vanadocyte), but not from that of the morula cells. In these experiments we used a combination of cell fractionation for purification of a specific type of blood cell, chromatography for extraction of vanadobin, and neutron activation analysis for determination of vanadium (Michibata and Uyama, 1990).

The present results clearly indicate that vanadobin is contained in the blood cells of all the ascidians examined. In general, ascidians belonging to the family Ascidiidae contain high levels of vanadium, whereas those belonging to Cionidae (Michibata, 1984; Michibata *et al.*, 1986b) and Corellidae (Hawkins *et al.*, 1983) do not always have such high amounts of vanadium. The amount of vanadium, even in vanadium-poor ascidians, is, however, thousands of times higher than that in other animals, including mammals. We may suppose, therefore, that other kinds of complexes with vanadium must be present in ascidian tissues. The occurrence of vanadobin in the blood cells of *Ciona intestinalis* and *Corella japonica* suggests that vanadobin is a universal complex in ascidian blood cells, and that its role is to accumulate and maintain vanadium.

One of the reasons that previous attempts to obtain vanadium-binding substances failed might be that the pH of the buffer solution used in the extraction of vanadium-binding substances was neutral. (There is utter confusion about the actual intracellular pH of ascidian blood cells.) Following Henze's first discovery of 1 *N* acidity (Henze, 1911), it was widely accepted that a homogenate of ascidian blood cells had a low pH value. However, Dingley *et al.* (1982) and Agudelo *et al.* (1983) claimed that the methods used in those early experiments gave spurious results because the cell interior containing the high levels of vanadium was most probably a highly reducing environment. Therefore, the possibility has not been eliminated that the intracellular redox potential, not the pH, was measured. When a new technique with an improved trans-membrane equilibrium of ^{14}C-labeled methylamine was used, the intracellular pH was neutral. Hawkins's group also measured nearly neutral pH in ascidian blood cells by means of a ^{31}P-NMR (Hawkins *et al.*, 1983; Brand *et al.*, 1987). Conversely, Frank *et al.* (1986) reported that the blood cells possess a pH value of 1.8 based on ESR spectrometry. These previous studies were, however, carried out on whole blood cells, without cell fractionation, and were focused on the green-hued morula cells which had been considered vanadocytes; not examined were the signet ring cells that have been newly identified as vanadocytes (Michibata *et al.*, 1987). In fact, we have recently demonstrated that the separated subpopulations of signet ring cells in *Ascidia ahodori, A. sydneiensis samea, A. gemmata,* and *Corella japonica* show low pH values ranging from 0.8 to 3.1, and that they also contain high amounts of vanadium, as determined by a combination of techniques involving cell fractionation, microelectrode measurements, and neutron activation analysis (Michibata *et al.*, 1990b). From this angle, it seems highly possible that vanadium binds with organic substances within the signet ring cells (strictly speaking, in the vacuole), and therefore, the previous failures in the extracting vanadium-

binding substances were due to conditions of pH. We also could not extract vanadobin under neutral conditions (data are not shown).

Roman *et al.* (1988) have recently extracted metal complexes with organic substances from the blood plasma of *Pyura chilensis* and *Ascidia dispar*. These substances seem to be heavier molecules than vanadobin because they eluted through Sephadex G-75. The blood plasma has been proposed to contain transferrin-like metalloprotein, although the biochemical roles in ascidian blood are still unknown (Roman *et al.*, 1988). On the other hand, because vanadobin is estimated to be a low molecular weight substance (about 1300), and the concentration of vanadium in the blood cells is 100 or more times greater than that in the blood plasma (Michibata *et al.*, 1986b; Roman *et al.*, 1988), these two types of metal binding substances probably have different roles in ascidian blood.

The highest concentration of vanadium was contained in the blood cells of *A. gemmata;* this corresponds to 4,000,000 times that of seawater (*cf.* Michibata, 1989). Strictly speaking, if almost all of the vanadium observed in the blood cells is contained in the vacuoles and binds with the vacuole membranes (Scippa *et al.*, 1988), its concentration should be much higher. Vanadobin, which can maintain the vanadium ion in the reduced form of +3 or +4 and has an affinity for exogenous vanadium ion (Michibata *et al.*, 1986a), must hold the key to resolving the specific accumulation of vanadium. Therefore, the next important study is to clarify the chemical structure of vanadobin.

Acknowledgments

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